Mechanisms of Ageing and Development, 13 (1980) 387–392

LOSS OF GENE REPRESSION ACTIVITY: A THEORY OF CELLULAR SENESCENCE

JAMES R. SMITH and CHARLES K. LUMPKIN, Jr.

W. Alton Jones Cell Science Center, Lake Placid, New York 12946 (U.S.A.)

(Received April 22, 1980; in revised form July 31, 1980)

SUMMARY

Recent evidence bearing on cellular senescence has come from cell fusion and clonal lifespan studies. To date, no model has been developed that is consistent with the recent findings.

The purpose of this paper is to present a mechanism for cell senescence that is qualitatively consistent with the experimental evidence.

INTRODUCTION

The observation that human diploid fibroblasts have a limited replicative lifespan *in vitro* [1] has generated a wealth of competing hypotheses [2]. Recent evidence bearing on cellular senescence has come from cell fusion [3] and clonal lifespan studies [4, 5]. The restrictions imposed by these data provide a backdrop against which specific mechanistic hypotheses can be tested or developed. The evidence accumulated to date is most compatible with the general idea of a genetic program of *in vitro* aging or terminal differentiation [6, 7]. Several models fall within this category [8, 9]; however, the majority lack specific mechanistic hypotheses [10]. Only recently have molecular mechanisms been proposed in partial explanation of a genetic theory for cell senescence [11, 12].

The purpose of this paper is to present a mechanism for cell senescence that is qualitatively consistent with the experimental evidence.

EXPERIMENTAL BACKGROUND

There are certain key experimental observations that we feel must be considered when proposing a theory to account for *in vitro* cellular aging. For convenience of reference these data are presented in the following form:

(1) Mass culture data: (a) Mass cultures lose replicative competence after a limited number of population doublings [1]. This is accompanied by a gradual increase in the fraction of non-dividing cells [13, 14]. (b) Spontaneously transformed cell cultures, cultures transformed by virus (SV-40) or chemical carcinogens and certain cultures established from tumors have an indefinite lifespan. (c) The individual cells within a mass culture are extremely heterogeneous with respect to doubling potential. The mean doubling potential of small samples of cells taken from a mass culture decreases with increasing doublings of the mass cultures [4, 5].

(2) Clonal culture data: (a) A bimodal distribution of intraclonal division potential of fibroblasts has been reported [4, 5]. (b) A bimodal distribution of division rates of serial subclones of fibroblasts and HeLa cells [7, 15] has been observed. (c) Variation has also been observed in the division potential of sister cells [5, 16, 17].

(3) Cell fusion data: (a) The fusion of young actively dividing cells to senescent cells produces cells unable to initiate DNA synthesis [18, 19]. (b) Transformed or cancerous cells fused to normal senescent cells produce either (i) initiation of DNA synthesis in the senescent nucleus, or (ii) a failure to initiate synthesis in either nucleus, depending on the immortal cell type [20, 21]. (c) Cells reconstructed from cytoplasts and karyoplasts indicate that the "senescence" character is dominant [22]. (d) Fusion of normal cells of varying lifespans produces tetraploid clones of intermediate lifespan [23].

The results from the mass culture studies are consistent with some type of multiple step process that results in a relatively precise timing of the loss of replicative competence. The heterogeneity observed in the clonal data indicates an intracellular stochastic element that must be reconciled with the consistent lifespan of the mass cultures. The variation in division potential of sister cells suggests some type of unequal partitioning during cytokinesis. The cell fusion studies indicate that senescent cells synthesize a substance that has the ability to diffuse through the cytoplasm and inhibit the initiation of DNA synthesis in an otherwise active nucleus. It should also be noted that, given the results from the fusion of two normal cells, the hypothesis that recessive mutations cause limited lifespan is unlikely [23].

In short, senescence appears to result from a diffusible inhibitor that is produced in a stochastic manner, yet results in a consistent timing for the loss of replicative competence of mass cultures of human diploid fibroblasts over a period of 100–200 cell generations [24].

In agreement with Stein and Yanishevsky [21], we would propose that cells can become "immortal" in at least two ways: (1) they lose the ability to produce active inhibitor gene products; for example, by somatic cell mutation or chromosome loss; (2) they make a new product that renders the inhibitor ineffective. As proposed previously [21], this would account for the results obtained from the fusion of senescent cells with various immortal cell lines [20, 21].

A STOCHASTIC MECHANISM

We have developed a tentative hypothesis that is consistent with the experimental findings discussed above which could account for *in vitro* cellular senescence. This

hypothesis is based on well-known biological processes or processes which have been postulated in other contexts. The main features of this hypothesis are listed below.

(1) Normal human cells contain a gene which codes for an inhibitor of DNA synthesis. This gene is not expressed in young cells. (2) The expression of the inhibitor is prevented by a repressor. (3) The gene coding for the repressor exists in multiple (perhaps tandem) copies. (4) The repressor gene copies can be lost through several mechanisms including imperfect replication or non-precise partitioning between sister cells. (5) When the number of repressor gene copies reaches a minimum threshold, the inhibitor gene is no longer repressed. (6) Once the inhibitor is expressed at a sufficient level, it is reinforced by a positive feedback mechanism.

From a procedural standpoint, we assume that young cells have an excess of repressor gene copies that function to prevent expression of the inhibitor. As cells divide, they distribute these repressor gene copies in a process that contains a stochastic component. This could be due to unequal crossing-over of chromosomal DNA or to non-precise physical partitioning of autonomous genetic elements. This process accounts for the variability observed in mass and clonal cultures and clones derived from sister human fibroblast cells. In other cell types the repressor gene copies may be distributed in a directed manner. If all or most of the copies were directed into one cell at each mitosis, this would result in stem cells and would account for the immortality of the germ line.

To model the loss of replicative competence in normal cells, we postulate the gradual loss of repressor gene copies throughout the *in vitro* lifespan. This could occur in two ways: (1) loss or inactivation of a certain *number* of copies at each cell generation; or (2) loss or inactivation of a certain *proportion* of copies at each generation. In the first instance the strongest effect will be found when the number of repressor gene copies is small. The second case would result in the reverse situation; *i.e.* a large number of gene copies would be inactivated when the number of repressor gene copies was large. Either of these processes would result in the gradual loss of proliferative potential with increasing *in vitro* population doublings. It is also possible that accidental loss of repression could occur even when several copies of the repressor gene are present. The probability of this occurrence would be inversely proportional to the number of repressor gene copies and would lead to an abrupt loss of division potential resulting in the observed bimodality of intraclonal proliferative potential [5]. Near the end of the *in vitro* lifespan of a culture, the interdivision time of the individual cells increases [15, 16]. This could result from low-level transient production of the inhibitor. During this stage the level of inhibitor production is not large enough to be "locked on" by a positive feedback mechanism. In the context of this model the increased *in vitro* lifespan obtained by glucocorticoids for human embryonic lung fibroblasts [25] or fibroblast growth factor for bovine endothelial cells [26] could result from either increased synthesis of repressor gene product, or increased affinity of the repressor for its active site in the presence of the growth factors.

Cell reconstruction experiments [22, 27] indicate that the nucleus is primarily responsible for determining the *in vitro* lifespan of human diploid fibroblasts. However, when the cytoplasm of an old cell is combined with the nucleus of a young cell, the

resulting cell has an impaired ability to proliferate [22]. These results suggest that the inhibitor gene and the repressor gene copies are both located in the nucleus.

If this is the case, the repressor genes could be either chromosomal or extra-chromosomal. If the repressor genes are located within the chromosomes a mechanism suggested by Johnson and Strehler [28] is applicable. This process involves the selective loss of tandemly duplicated genes through the deletion of hairpin loops caused by mis-registered pairings. This theory provides for both the clonal variation and for the gradual loss of repressor genes. A similar idea has been presented by Tartoff [29] who sees unequal sister chromatid exchange both as the result of misregistered pairings and as the cause of ribosomal RNA gene amplification. In this context the relatively high basal level of sister chromatid exchange found in human diploid fibroblasts is suggestive [30]. Unequal crossing-over between homologous chromosomes (somatic recombination) has been suggested as the basis for cellular senescence [31]. Good [12] has proposed that the loss of DNA inherent in the replication process, could also provide a mechanism for cellular aging (theory of marginotomy).

In the classical case of ribosomal RNA gene amplification in amphibian oocytes, the genes are located extra-chromosomally in the nucleoli (or episomes) [32–34]. Recent work on human oocytes indicates a four-fold increase in ribosomal DNA which correlates with nucleolus-like structures [35]. If the repressor genes behave in a manner analogous to ribosomal RNA genes then unequal partitioning during cytokinesis would explain the clonal lifespan variations. The loss of repressor gene copies (DNA) posited by this model is analogous to the finding of an age-related decrease in hybridizable rDNA loci in canine brain, heart and skeletal muscle and heart, hippocampus and sensory motor cortex of aged human subjects [36–38]. Again, Strehler's selective loss hypothesis may be applicable. The importance of multiple gene copies as they relate to a theory of *in vitro* aging has been cogently argued by Ohno and Nagai [10].

At this time there is at least one other biological process which is consistent with this model. That is the instability of gene-amplified drug resistance [39]. The resistance of mouse cells to methotrexate results from high rates of synthesis of dihydrofolate reductase and correspondingly high numbers of reductase genes in cells selected by growth in the presence of methotrexate. In many of the resulting cultures the resistance is gradually lost when the cells are grown in the absence of the drug. This combination of initial amplification followed by sequential loss fits the model proposed here very well.

We should also consider the possibility that the repressor gene copies are extra-nuclear. In this context, the analogy between plasmid inheritance in bacteria and mitochondrial inheritance in mammalian cells deserves mention. The loss of repressor gene copies could be due to the failure to replicate before division, while the intraclonal variability would be due to random assortment of repressor gene copies into sister cells at cytokinesis. Evidence for the occurrence of these processes in bacteria has been produced and modeled [40].

The possible mechanism presented here strongly points up the need for more data for future tests of this model. Fusion of clones of cells with disparate lifespans could produce evidence against which this hypothesis can be tested. Quantitative computer

simulation of this model is now in progress and will allow future tests relevant to *in vitro* aging, especially concerning the proliferative potential of hybrid clones of the kind mentioned above as well as to the loss of methotrexate drug resistance.

Furthermore, this hypothesis predicts the decrease of a specific protein – the repressor gene product – during *in vitro* aging and the appearance of a new protein in senescent cell cultures – the "inhibitor". If the repressor gene product can be identified then the decrease in repressor gene copies with age can be verified using RNA–DNA hybridization techniques. It is also possible that the mechanism of control of the DNA synthesis inhibitor gene is not unique. In that case we would expect to find an increasing proportion of human diploid fibroblasts in senescent cultures producing atypical products, such as hemoglobin, albumin, *etc.*

ACKNOWLEDGEMENTS

This work was supported by NIH Grants AG00338, T32-AG00043 and the W. Alton Jones Foundation.

REFERENCES

1 L. Hayflick and P. S. Moorhead, The serial cultivation of human diploid cell strains. *Exp. Cell Res., 25* (1961) 585–621.
2 P. I. Good, Aging in mammalian cells population – review. *Mech. Ageing Dev., 4* (1975) 339–348.
3 T. H. Norwood, Somatic cell genetics in the analysis of *in vitro* senescence. In E. L. Schneider (ed.), *The Genetics of Aging,* Plenum Press, New York, 1978, pp. 337–371.
4 J. R. Smith and L. Hayflick, Variation in life-span of clones derived from human diploid cell strains. *J. Cell Biol., 62* (1961) 48–53.
5 J. R. Smith and R. G. Whitney, Intraclonal variation in proliferative potential of human diploid fibroblasts: stochastic mechanism for cellular aging. *Science, 207* (1980) 82–84.
6 J. Morrow and C. Garner, An evaluation of some theories of the mechanism of aging. *Gerontology, 25* (1979) 136–144.
7 G. M. Martin, C. A. Sprague, T. H. Norwood and W. R. Pendergrass, Clonal selection, attenuation and differentiation in an *in vitro* model of hyperplasia. *Am. J. Pathol., 74* (1974) 137–154.
8 P. I. Good and J. R. Smith, Age distribution of human diploid fibroblasts – stochastic model for *in vitro* aging. *Biophys. J., 14* (1974) 811–822.
9 T. B. L. Kirkwood and R. Holliday, Commitment to senescence – model for finite and infinite growth of diploid and transformed human fibroblasts in culture. *Biophys. J., 53* (1975) 481–496.
10 S. Ohno and T. Nagai, Genes in multiple copies as the primary cause of aging. In D. Bergsma, D. E. Harrison and N. W. Paul (eds.), *Genetic Effects on Aging,* Alan R. Liss Inc., New York, Vol. XIV, No. 1, 1978, pp. 501–514.
11 S. Shall and W. D. Stein, A mortalization theory for the control of cell proliferation and for the origin of immortal cell lines. *J. Theor. Biol., 76* (1976) 219–231.
12 P. I. Good, Stochastic model for *in vitro* aging. 2. Theory of marginotomy. *J. Theor. Biol., 64* (1977) 261–275.
13 V. J. Cristofalo and B. B. Sharf, Cellular senescence and DNA-synthesis thymidine incorporation as a measure of population age in human diploid cells. *Exp. Cell Res., 76* (1973) 419–427.
14 G. S. Merz and J. D. Ross, Viability of human diploid cells as a function of *in vitro* age. *J. Cell. Physiol., 74* (1969) 219.
15 A. O. Martinez, T. H. Norwood, J. W. Prothero and G. M. Martin, Evidence for clonal attention of growth potential in HeLa cells. *In Vitro, 14* (1978) 996–1002.

16 P. M. Absher and R. G. Absher, Clonal variation and ageing of diploid fibroblasts – cinematographic studies of cell pedigrees. *Exp. Cell Res., 103* (1976) 247.
17 E. Bell, D. Levinstone, S. Sher, L. Marek, C. Merrill, I. Young and M. Eden, An interactive computer system for the analysis of cell lineages. *J. Histol. Cytol., 27* (1976) 458–462.
18 T. H. Norwood, W. R. Pendergrass, C. A. Sprague and G. M. Martin, Dominance of senescent phenotype in heterokaryons between replicative and post-replicative human fibroblast-like cells. *Proc. Natl. Acad. Sci. U.S.A., 71* (1974) 2231–2236.
19 R. M. Yanishevsky and G. H. Stein, Entry into S-phase is inhibited, but on-going DNA- synthesis continues in young human diploid cells (HDC) fused with senescent HDC. *J. Cell Biol., 79* (1978) 398.
20 T. H. Norwood, W. R. Pendergrass and G. M. Martin, Reinitiation of DNA synthesis in senescent human fibroblasts upon fusion with cells of unlimited growth potential. *J. Cell Biol., 64* (1975) 551–556.
21 G. H. Stein and R. M. Yanishevsky, Entry into S-phase is inhibited in two immortal cell lines fused to senescent human diploid cells. *Exp. Cell Res., 120* (1979) 155–165.
22 A. L. Muggleton-Harris and L. Hayflick, Cellular aging studied by the reconstruction of replicating cells from nuclei and cytoplasms isolated from normal human diploid cells. *Exp. Cell Res., 103* (1976) 321–330.
23 H. Hoehn, E. M. Bryant and G. M. Martin, Replicative life-spans of euploid hybrids derived from short-lived and long-lived human skin fibroblast cultures. *Cytogenet. Cell Genet., 21* (1978) 282–295.
24 P. I. Good, Subcultivations, splits, doublings, and generations in cultures of human diploid fibroblasts. *Cell Tissue Kinet., 5* (1972) 319–323.
25 V. J. Cristofalo, Hydrocortisone as a modulator of cell division on population life-span. In V. J. Cristofalo (ed.), *Explorations in Aging,* Plenum Press, New York, 1975, pp. 57–80.
26 D. Gospodarowicz, J. S. Moran and D. L. Braun, Control of proliferation of bovine vascular endothelial cells. *J. Cell. Physiol., 91* (1976) 377–386.
27 W. E. Wright and L. Hayflick, Nuclear control of cellular aging demonstrated by hybridization of anucleate and whole cultured normal human fibroblasts. *Exp. Cell Res., 96* (1975) 113–121.
28 R. Johnson and B. L. Strehler, Loss of genes coding for ribosomal RNA in ageing brain cells. *Nature, 240* (1972) 412–414.
29 K. D. Tartof, Unequal mitotic sister chromatid exchange as mechanism of ribosomal RNA gene modification. *Proc. Natl. Acad. Sci. U.S.A., 71* (1974) 1272–1276.
30 E. L. Schneider and R. E. Monticone, Aging and sister chromatid exchange. 2. Effect of *in vitro* passage level of human fetal lung fibroblasts on baseline and mutagen-induced sister chromatid exchange frequencies. *Exp. Cell Res., 115* (1978) 269–276.
31 J. R. Callahan, Genetic recombination in mammalian somatic cells – Brief note. *Mech. Ageing Dev., 8* (1978) 281–284.
32 D. C. Brown and I. B. Dawid, Specific gene amplification in oocytes. *Science, 160* (1968) 272–280.
33 F. Ritossa, Procedure for magnification of lethal deletions of genes for ribosomal-RNA. *Nature, 240* (1972) 109–111.
34 F. Ritossa, Crossing over between X and Y chromosomes during ribosomal DNA magnification in *Drosophila melanogaster. Proc. Natl. Acad. Sci. U.S.A., 70* (1973) 1950–1954.
35 D. J. Wolgenmuth, G. M. Jagiello and A. S. Henderson, Quantitation of ribosomal RNA genes in fetal human oocyte nuclei using rRNA: DNA hybridization *in situ. Exp. Cell Res., 118* (1979) 181–190.
36 R. Johnson, C. Chrisp and B. L. Strehler, Selective loss of r-genes during aging of post mitotic tissues. *Mech. Ageing Dev., 1* (1972) 183–198.
37 R. Johnson and B. L. Strehler, Loss of genes coding for ribosomal-RNA in aging brain cells. *Nature, 240* (1972) 412–414.
38 L. K. Johnson, R. W. Johnson and B. L. Strehler, Cardiac hypertrophy, aging and changes in cardiac ribosomal-RNA gene dosage in man. *J. Mol. Cell. Cardiol., 7* (1975) 125–133.
39 R. T. Schimke, R. J. Kaufman, F. W. Alt and F. Kellems, Amplification and drug-resistance in cultured murine cells. *Science, 202* (1978) 1051–1055.
40 J. E. Dowman, Implication of stochastic inheritance. *J. Theor. Biol., 39* (1973) 55–72.